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Effect of dietary resistant starch on inhibition of preneoplasia in azoxymethane-induced rodent models

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Effect of dietary resistant starch on inhibition of preneoplasia in azoxymethane-induced rodent models

by

Bridget Nelson

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

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Major: Toxicology

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ABSTRACT

Diet choices can play a role in disease incidence, especially diseases associated with the digestive system, such as Irritable Bowel Syndrome, Crohn's disease, and colorectal cancer. Starch is a large component of the the human diet and the non-digestible portion, referred to as dietary fiber, has been implicated as a factor that decreases the risk for colorectal cancer. Modified or naturally occurring resistant starches share similar digestion resistant properties as dietary fiber and may produce similar protective effects. Two different animal models, F344 rats and A/J mice, were used to compare preneoplasia inhibition when fed one of three resistant starch diets or a control cornstarch (CS). The resistant starches used included a native high amylose starch (HA7), a high-amylose chemically modified with octenyl succinic anhydride (OS-HA7), and a high-amylose complexed with stearic acid (SA-HA7). Two common preneoplasia are aberrant crypt foci (ACF) and mucin-depleted foci (MDF). Azoxymethane (AOM), a chemical carcinogen, was used to induce the preneoplasia in the rodent models by means of weekly intraperitoneal injections; rats received two weekly injections of 15mg AOM/kg body weight, mice received four weekly injections of 7.5mg AOM/kg body weight. Saline injections were used as a control. Experimental diets were fed for ten weeks following the last carcinogen injection. Antibiotics were also administered to a few of the rats to determine the potential effects on preneoplasia inhibition when fed resistant starch diets. In both animal studies, the animals treated with AOM and fed the SA-HA7 diet developed the greatest number of ACF. While the SA-HA7 diet had the greatest number of ACF, it subsequently had the fewest MDF present. The animals fed the resistant starch diets that

developed the fewest ACF were different between the two animal studies; OS-HA7 fed rats and HA7 fed mice. Analysis by two and three-way ANOVAs determined that there was no diet effect experienced in either study for ACF. The MDF results suggest that MDF may not be a reliable or relevant biomarker for colorectal cancer as the greatest number of MDF were observed in rats fed the CS diet and treated with saline and administered antibiotics. General conclusions from this study recommend further studies to identify a reliable biomarker and to determine the long term effects of resistant starch intake on the inhibition of colon tumors.

CHAPTER 1: GENERAL INTRODUCTION

Introduction

Resistant starch (RS) escapes normal digestion in the small intestine and is instead fermented by gut microbes in the large intestine. The resistant starch acts as a fermentable substrate for the production of short chain fatty acids (SCFA) which are believed to provide beneficial effects for the colon such as fecal bulking, increasing blood flow in the colon, and improved absorption of water and minerals [1]. Due to the amount of starch that passes on to be fermented and the similarities in other physiological functions, it has been suggested that RS may act as a dietary fiber [2].

Resistant starch can be found in a variety of natural plant sources and is classified into four types. Type 1 RS is physically inaccessible starch. Type 2 is naturally resistant to α -amylase digestion. Type 3 refers to retrograded starch commonly found in cooked-then-cooled foods. Type 4 RS refers to chemically modified starches. A common chemical modification is done by esterification of a naturally high amylose starch using octenyl succinic anhydride (OS-HA7) to chemically modify a hydrophobic component to the starch. Type 5 is novel RS that complexes the high amylose starch with stearic acid (SA-HA7), a lipid, which alters the starch structure to make it more resistant to digestion.

Accumulated studies have suggested and supported the benefits of RS in colorectal disease such as CRC and inflammatory bowel disease [3]. The most common SCFA produced are acetate, propionate, and butyrate. All of these SCFA, especially butyrate, are implicated in the prevention of colon carcinogenesis [3]. Butyrate is an important byproduct of RS fermentation as this particular SCFA may play a specific role in

regulating the initiation of CRC [1]. Specifically, butyrate can decrease CRC cell proliferation and induce apoptosis as well as increase normal cell proliferation and cell differentiation [4].

The study in this thesis investigated the potential inhibitory effect two modified RS: a modification with octenyl succinic anhydride and a modification with stearic acid. The inhibitory effect was determined by the incidence of preneoplastic lesions aberrant crypt foci (ACF) and mucin-depleted foci (MDF) in azoxymethane treated mice and rat subjects. These colonic preneoplasia have been extensively studied. ACF are characterized by abnormal colonic crypt shape and increased luminal space. MDF are noted for the lack of mucin production in which normal mucin levels aid in fecal material transport along the colon. Older studies suggest that RS either enhanced ACF or had no effect on colon carcinogenesis [5, 6, 7] while newer studies propose a protective effect by RS in a dose-dependent manner [3, 8, 9]. MDF, however, have been proposed as a better biomarker for colon carcinogenesis but further studies are needed to better understand the correlation [10]. It is generally hypothesized that diets containing resistant starch will show greater inhibition of azoxymethane carcinogen-induced preneoplastic lesions in the colons of rodents. For rats, it was hypothesized that the SA-HA7 diet would provide the greatest inhibition of colonic preneoplasia due to its digestibility resistance. It was also hypothesized that mice would exhibit the same trend of SA-HA7 prevention of preneoplasia and reproduce similar results as the rat study.

Thesis Organization

This thesis attempts to enhance our current knowledge of diet and cancer prevention through the feeding of dietary resistant starch to animals and analyzing the inhibitory effect on colonic preneoplasia. The first chapter in this thesis contains a general introduction with a literature review. The second chapter contains a manuscript titled “Effect of dietary resistant starch on inhibition of colonic preneoplasia in azoxymethane-induced rodent models” that will be submitted to a food science and carcinogenesis related journal to be determined. The third chapter contains general conclusion obtained from the animal studies listed in the second chapter. Literature sources cited in each chapter appear at the end of the appropriate chapter in the order they appear.

This study would not have been possible without the help of a few key contributors. My contribution consisted of experimental design and set up, daily animal care, starch processing, and animal sacrifice and tissue analysis for both rat and mouse studies. The Jane lab members, particularly Yongfeng Ai, demonstrated and oversaw the resistant starch production in the pilot plant as well as mentored in resistant content analysis and anything starch related. Nicole Cray contributed to the experimental design and set up, daily animal care, and animal sacrifice and tissue analysis for the mouse study.

CHAPTER 2: LITERATURE REVIEW

Colorectal Cancer

Colorectal cancer (CRC) is one of the most common cancers in the United States for both men and women with the incidence being slightly higher in men. The American Cancer Society predicts approximately 102,480 new cases of colon cancer and 40,340 new cases of rectal cancer for 2013 [11]. The overall number of colon and rectal cancers has declined steadily over the past 20 years due to advances in early screening and excision procedures [97, 98]. Even with these advances, CRC remains the third highest in cancer incidences and the second leading cause of cancer deaths. It is estimated that individuals throughout the world consuming a “Western diet” high in fat and low in essential vitamins may have a greater risk for colon cancer, with the United States having the highest incidence [12, 96]. African Americans have also been found to have the highest incidence and mortality rates for CRC compared to all other races, though the reason is not yet understood [99]. Individuals of Eastern European Jewish descent have also been named as one of the cultural groups with a very high rate of incidence of CRC due to frequent gene mutations within this heritage [11].

Colorectal cancer refers to uncontrolled cell growth in the colon or rectum areas of the gastrointestinal tract [11]. Cancers of the colon and rectum are often initiated in the epithelial linings of the bowel and progress to the malignant state termed adenocarcinomas, which is the most common form of CRC as well as the best characterized [56]. Lymphomas and carcinomas can also be present but are less frequent in the bowel than adenocarcinomas. Preneoplastic lesions refer to abnormalities in the

colonic epithelium that precede the development of a tumor. These lesions are common in CRC but studies have suggested that a very low percentage of these precancerous growths actually progress towards malignant cancers [14, 15]. Epidemiological studies have determined several risk factors that may play a role in CRC incidence including age, gender, family history of CRC and other bowel diseases, inactivity, obesity, heavy alcohol consumption and smoking [13]. Most new CRC cases are due to lifestyle impacts rather than genetic history with an overall risk of 1 in 20.

Carcinogenesis can occur by sporadic initiation as a result of a lifestyle impact, hereditary genetics, or genetic mutations. With the increase in number and frequency of common risk factors, sporadic CRC cases are more common than genetic cases, accounting for nearly 75% of all CRC related cases [16]. Soravia et al. identifies and describes two common hereditary conditions of CRC: Familial Adenomatous Polyposis (FAP) and Hereditary Nonpolyposis Colorectal cancer (HNPCC). FAP patients develop hundreds of polyps in the colon at a young age and have fully developed cancer by age 40 in nearly all FAP patients. Those with FAP are thought to have an increased risk for other related cancers such as small intestine. HNPCC, however, does not develop as many polyps as those with FAP but still have fully developed cancer around age 40, with a slightly lower incidence risk than FAP. HNPCC patients also have increased risk for associated cancers but instead have this higher risk for uterine or stomach cancers [16].

Both hereditary conditions exhibit an inherited germline mutation but differ in the mutated gene. Heyer et al discusses differences between the two hereditary conditions FAP is an inherited mutation of the adenomatous polyposis coli (APC) gene. The APC gene

is responsible for tumor suppression in healthy individuals. The mutation associated with FAP accounts for 1% of the total CRC cases. HNPCC is an inherited mutation or defect in the MutL homolog 1 (MLH1) or MutS protein homolog 2 (MSH2) genes both responsible for DNA mismatch repair functions [45].

Diet choices have been implicated as one of the biggest factors linked to increased CRC risk in non-hereditary cases. Our studies use a chemically-induced animal model to determine the effect of diet on the inhibition of preneoplastic lesions in the colon. Azoxymethane is most commonly used as the chemical carcinogen because its mutations, primarily with Kras and beta catenin, are also found in sporadic cases [83-85]. The mutation in the APC gene is found in both sporadic and hereditary cases of CRC, but the APC mutations in FAP cases account for only 1% of all CRC cases [45] therefore identifying an APC mutation as a sporadic CRC mutation. This distinction confirms the rationale behind using a chemically-induced model as an appropriate model for sporadic CRC incidence.

Diet and Lifestyle

Most colorectal cancer cases are induced by environmental factors such as diet and lifestyle rather than hereditary genetics [16]. Lifestyle factors such as reduced activity level, alcohol consumption, smoking, and obesity can increase the occurrence of CRC [18-20]. One particular study conducted by Fu et al 2012 focused on lifestyle impacts on 6307 patients (3764 control and 2543 adenomas only, hyperplastic polyps only or both) in Tennessee between February 2003-March 2010. Three primary non-dietary related factors were analyzed: smoking, BMI, and regular nonsteroidal anti-inflammatory drugs

(NSAIDs). This study found that all of these factors were independently associated with risk of colorectal polyps with smoking and high BMI in particular are well characterized risk factors for CRC. Smoking had a higher association with hyperplastic polyps than adenomas while the remaining lifestyle factors investigated had similar strength in association between the two types of colorectal polyps. Regular NSAID use was found to be associated with a decreased risk of CRC because of its inhibitory effect of COX-2 enzyme which has been implicated in colorectal carcinogenesis [100]. The results of this study suggested that early prevention of CRC can be greatly impacted by lifestyle modifications [17].

Diet also plays an important role in CRC risk. Dietary fiber has been widely accepted as a benefit to colon health and has been implicated in the prevention of CRC [4] This concept, however, has come under scrutiny as some studies conducted to examine the possible protective effects of dietary fiber on CRC have yielded contradicting results. While there are studies suggesting no protective effect, there is a greater accumulation of data suggesting a decreased risk of CRC with an increased intake of dietary fiber. A study conducted by Dahm et al. used 579 CRC patients and 1996 control patients and 4-7 day food diaries to assess the effect of dietary fiber on CRC recurrence risk. It was found that absolute fiber intake and fiber intake density were inversely associated with risk of CRC. This study also suggested the differences in results observed in other studies could be attributed to differences in study design [21]. Park et al conducted a pooled analysis of prospective cohort studies for dietary fiber and CRC risk. Thirteen cohort studies including 725, 628 participants (men and women) used study

specific food frequency questionnaires and had follow-up times ranging from 6-20 years. Overall, increased dietary fiber intake was inversely associated with CRC risk with age-adjusted models but the relationship was no longer significant after adjusting for other CRC risk factors, such as red meat intake and alcohol consumption [22].

Many developed countries, especially the United States, eat foods that are part of “Western diets” which are characterized by high intakes of red meat, fats, and sugars. The number of red meat and CRC risk studies have grown over the past few decades and controversy surrounds the relationship between red meat intake and CRC risk. One study from 1994 suggested that men who consumed red meats, especially beef, pork, and lamb, more than 5 times per week as a main dish component had a higher CRC risk than men who ate red meat products less than once per month on average [23]. A more recent study from 2009, however, has found no significant evidence that an association between animal fat and protein intake and CRC risk exists based on a 6 prospective cohort study analysis established on a dose-response study design. This study explains that overall the association between meat consumption and CRC risk was weak due to varying tumor sites and the inability to separate meat consumption effects from other CRC risk factors’ effects [24].

Starches

Starch is a carbohydrate generated by most green plants and is a common component of the human diet. Staple foods that contain starch include potatoes, corn and rice. Starch is a polysaccharide of glucose subunits that is composed of two types of molecules: linear, helical amylose and branched amylopectin. The linear glucose subunits

are connected by α -1,4 glycosidic linkages while the branch points of amylopectin are connected by α -1,6 glycosidic linkages. Amylopectin is regularly found in a crystalline form while amylose is amorphous.

Starches are generally consumed cooked, especially in the human diet. Gelatinization uses water and heat to break down the starch molecules to allow for more water binding sites, causing the starch granules to swell. This heating and swelling causes the structure of the starch to change from crystalline to amorphous and causes a random arrangement of the amylose and amylopectin chains. Gelatinization temperature is mainly affected by amylopectin structure and amylose content. The more amylose present, and therefore more compact structure, will have a high gelatinization temperature.

Retrogradation is also an irreversible reaction that takes place after gelatinization during a cooling step when amylose and amylopectin chains realign themselves to a more crystalline structure, forming a gel-like substance. Amylose retrogrades faster than amylopectin due to the hydrogen-bridges that are formed after rearrangement. Some modifications of native starches include a lipid moiety that increases retrogradation time. Lipids complex with amylose and the resulting structure prevents retrogradation of the trapped starch granules [31].

Enzyme hydrolysis is also an important characteristic of starches. Hydrolysis breaks down starch to monomer glucose units which are used in biochemical pathways for energy for plants, animals, and humans. Many factors affect the rate of enzyme hydrolysis of starch including amylose and amylopectin content, starch granule size, and

presence of lipids. Enzyme hydrolysis is slower for native, ungelatinized starches as well as large starch granules [32]. Amylase is present in the saliva in humans and is the first step of digestion. Amylase cleaves the starch chain at the α -1,4 glycosidic linkages of linear amylose chains. Amyloglucosidase is responsible for the cleavage of α -1,6 linkages commonly found in amylopectin.

Resistant starch is a type of starch that escapes normal digestion in the small intestine and is instead fermented in the large intestine by the gut microbiota. The major fermented products are known as short chain fatty acids (SCFA) and studies have suggested their CRC inhibiting potential [29-30]. RS can be classified into 4 types. Type 1 RS is starch that is physically inaccessible due to a protein or cell wall material that provides an impermeable barrier to enzymes and water. Whole grains and legumes are types of RS1 that have this barrier to make it resistant to hydrolysis and swelling. Type 2 RS is a starch that holds a native semi-crystalline structure when uncooked. When subjected to gelatinization, the structure breaks down to an amorphous configuration which is more susceptible to enzyme hydrolysis and digestion. Foods that contain the RS2 type are green bananas, raw potatoes, and native high amylose starches. Type 3 RS is classified as retrograded starch found in cooked-then-cooled foods such as potatoes. Type 4 RS is a class of chemically modified starches. Modifications to native starches can decrease enzyme and water accessibility to the starch granules, making it more resistant. The most common type of modification is chemically modifying a high-amylose native starch (RS2) with octenyl succinic anhydride, a hydrophobic moiety [33]. A novel type of RS, referred to as RS5 is a high-amylose-lipid complexed starch. This new starch is thought

to be the most resistant to digestion as the many amylose chains are intermixed with lipid constituents that block enzyme binding sites, making it more resistant to digestion. The added lipid also reduces water solubility [29, 34].

Resistant starch has been implicated as a benefit in a few of the most common human health problems. Obesity is a major health concern, especially in cultures consuming a “Western” diet which is high intakes of red meat, fat, and refined grains. The addition of a slowly digestible starch like RS to the diet is expected to help moderate the rate of digestion and increase satiety leading to a potential decrease in caloric intake. The glucose and insulin response is also important for diabetes control. Ten men given a 50g sample of starch containing either 0% or 54% RS found that the RS sample significantly reduced blood glucose and insulin concentrations [35]. RS has also been associated with decreasing the risk of cardiovascular disease. Diets containing 25% raw potato starch lowered plasma cholesterol and triglyceride levels in rats [2].

The resistant starch that makes it to the colon is fermented to produce short chain fatty acids (SCFA) which have been suggested in the prevention of colon cancer. Butyrate, a SCFA produced, inhibits initiation of malignant cells in the colon [36]. Studies also suggest that diets containing RS can inhibit preneoplastic lesions and tumors. A previous short term study found that rats fed RS, particularly a high amylose starch, had decreased aberrant crypt foci (ACF), a common preneoplastic lesion in the colon. Another study fed 0%, 10% or 20% high amylose diets to rats for 25 weeks following an azoxymethane (AOM) carcinogen treatment and found that diets containing RS, at both doses, decreased the incidence of adenocarcinomas compared to the 0% high amylose diet [37].

In our studies, the focus of RS benefits is with the prevention or inhibition of preneoplasia in two different animal models, rats and mice. In both experiments, the starches underwent gelatinization from the cooking process and fed fresh to the animals to prevent retrogradation and other potential adverse effects of storage conditions on the digestibility resistance of the gelatinized starches.

Short Chain Fatty Acids

Short chain fatty acids (SCFA) are the products of microbial fermentation in the colon following ingestion of slowly digesting fibers or resistant starches. The most common SCFA produced are acetate, butyrate, and propionate with acetate occurring in the greatest amount over the other two common SCFA [38].

Production of SCFA in the colon has been suggested to have potential benefits for lowering CRC risk. Increased caecal and fecal weights have been implicated in lower risk for CRC as the RS is only partially fermented, and the undigested, residual polysaccharides absorb water which adds to the total weight and aids in preventing diarrhea [29, 30]. SCFA have also been implicated in decreased fecal transit time. The decreased time has the potential to decrease the contact time between the colon and carcinogen as well as dilute the fecal constituents [39, 40]. Since SCFA are weak acids, the colon pH is decreased by large amounts of SCFA produced in the colon. The more acidic nature of the intestine could prevent the growth of pathogenic bacterial species such as *E.coli* and *Salmonella* [38].

Dietary fibers and resistant starches have been reported to inhibit CRC aspects like preneoplastic lesions and tumors. When fed RS diets, rats experienced a decrease in ACF

and MDF numbers as well as an increase in total SCFA concentration [4]. Studies conducted in animal models have suggested a strong protective effect of dietary fibers and resistant starches against tumors in the colon. Epidemiologic studies, however, have found that protective effects, if any, were weak [38].

While acetate is consistently produced in the highest concentrations, butyrate is implicated as the SCFA with the greatest potential of CRC inhibition. Butyrate is an important energy source for normal functioning colonocytes. The presence of this energy source increases colon mucosal integrity, protects against DNA damage, and detoxifies carcinogenic agents [30]. Butyrate is of importance because of its known apoptotic, differentiation, and anti-proliferative nature [38]. A few mechanisms of CRC prevention by butyrate have been proposed. CRC is largely initiated by epigenetic factors such as diet. Butyrate inhibits histone deacetylase which is an epigenetic regulator that affects cell cycle and apoptosis events [30]. Butyrate has also been proposed to induce gene expression related to detoxification of oxidative stress products [41].

While this thesis does not directly study the effects of SCFA on preneoplasia inhibition in animal models, butyrate and other SCFA still play an important part in understanding the complexities of RS and CRC risk.

Animal Models

Animal models have proven useful for the study of human diseases, particularly colorectal cancer (CRC). These models, usually rats or mice, help to identify many different aspects of cancer including pathways associated with cancer initiation and progression, tumor incidence and malignancy, and preventative or therapeutic measures.

There are genetically modified animals, like the APC^{min} mouse line, that are used to mimic the tumorigenesis of hereditary CRC diseases in humans. There are also animal models bred to be specifically susceptible to a chemically-induced CRC state to study sporadic tumorigenesis, such as the A/J mouse line which is very sensitive to induction of preneoplastic lesions by azoxymethane [43]. Currently, rats and mice are the two species that are used for many CRC studies, with mice being used more frequently over rats due to the higher frequency of distal colon tumors [42].

Animal models provide several very distinct advantages: the increasing number of genetic models (transgenic, knockout), the availability of genetic information on individual animal lines, and the existence of recombinant inbred mouse panels [42]. Animal models also permit the study of new potential carcinogens and effectiveness of treatments that are otherwise unable to be studied in human subjects. Animal models have similar anatomy and physiology to that of humans and a shorter life span, making studies fast and reproducible. While there are many similarities between humans and animals, they are not exactly the identical, meaning that information received from animal studies needs to be carefully interpreted and may not always be completely applicable to humans. For CRC specifically, tumors produced in these animals often lack the invasiveness and metastatic nature to completely mimic human CRC. While the correlation between animals and humans may not be completely on target, animal studies have allowed for great advancement of various research areas and the use and benefit of animal subjects cannot be understated.

Since CRC is largely influenced by diet and other epigenetic factors, chemically induced animals provide a model that effectively mimics nonhereditary colorectal tumorigenesis. Azoxymethane (AOM) is the most common chemical carcinogen used to initiate CRC events in mice and rats. AOM does not have a direct effect on DNA but is instead metabolized to methylazoxymethanol (MAM) which induces DNA-reactive adducts and alkylation of guanine in DNA macromolecules in the liver and colon [44]. AOM itself is a downstream metabolite of 1, 2-dimethylhydrazine (DMH) which is also commonly used in animal CRC studies. While DMH is often used, AOM is more potent, more stable for injections than DMH, and does not produce the high level of inflammation as DMH. Therefore, AOM is used more often than DMH in colon carcinogenesis models [42]. Rosenberg et al. observe that even though the mouse lines exhibited various susceptibility responses to AOM, tumors found in all strains had similar morphologies.

Heterocyclic amines have also been used to induce tumors in rodents. One chemical in this class, 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP), utilizes a multiple organ specific model where other organs in addition to the colon are affected, such as the mammary gland and prostate tissues [42]. PhIP is rapidly absorbed out of the alimentary canal and distributed throughout the body. Even with the distribution to a wide range of tissues, the tumor incidence caused by PhIP is still very low, even after a 52 week exposure [51]. Another group of researchers also found that PhIP can induce a larger amount of tumors when fed in combination with a high fat diet based off of a control AIN-93 diet with added hydrogenated vegetable oil to supply 59% of total calories

from fat [25]. In all of the studies using PhIP to induce tumorigenesis in the colon, the overall tumor incidence was still quite low.

Dextran sodium sulfate (DSS) is a chemical that induces inflammation in rodent models. Induction by DSS is used to mimic inflammatory bowel diseases in humans, especially ulcerative colitis and Crohn's disease [48-50]. DSS is often administered in combination with another chemical carcinogen, most often DMH or AOM. The combination of DSS with AOM has been found to act as a two-stage carcinogenesis model where AOM acts as the initiator and DSS acts as the promoter for tumorigenesis in the colon of the animal models [42,47,48]. DSS is a strong promoting agent but needs additional periods of time and repeated exposure to induce colitis in rodent models. Tumor frequency from this tumorigenesis model is usually much lower than other chemically induced models [48]. Okayasu et al studied the effects of 0 (n=5) and 3% DSS (n=25) in drinking water in mice and only two tumors were found among both groups. DSS has been found to induce tumors when administered as the sole chemical agent but the tumor incidence is even lower than that of the combination scenario [42, 49].

Chemical carcinogens influence tumorigenesis in the colons of animals and each specific animal strain has a different susceptibility or resistance to carcinogens. The genetic background of the animal model is a significant component of CRC and other organ-specific carcinogenesis [42]. Factors that influence sensitivity to chemical carcinogens include: rates of activation and detoxification of the carcinogen, efficiency of damage repair, and a balance between cell apoptosis and proliferation [42, 45, 46]. In the case of AOM, the mouse strains A/J, P/J, STS/A, and ICR/Ha are very susceptible to AOM

induced preneoplasia and tumorigenesis, Balb/cHea and SWR/J lines are moderately susceptible to AOM, and AKR/J, DBA/2J, and C57BL mouse lines are resistant to the effects of AOM induction of tumorigenesis [42,43].

Our studies used the AOM carcinogen induction model in F344 rats and the AOM-sensitive A/J mouse line. The chemical induction model was chosen over a genetic model because our hypothesis is that diet plays a major factor in the initiation and development of colonic preneoplasia and tumorigenesis. Repeated use of this model has validated its use as an effective AOM-induced CRC model. This particular model was first studied by Papanikolaou et al in 1998 and since then numerous research groups have found the A/J mouse to be susceptible to AOM treatments in the formation of preneoplasia or tumorigenesis in the colon [25-28] Our lab has conducted three A/J mouse with AOM-induction studies and have consistently observed preneoplasia formed in each of the studies.

Lesions

Aberrant crypt foci (ACF)

Many researchers have identified and accepted aberrant crypt foci (ACF) as a common precancerous lesion found exclusively in the colon in both people with and without colorectal cancer (CRC) [14]. ACF were first characterized by Bird by use of a methylene blue staining procedure [59]. ACF feature a few distinct characteristics that distinguish them from normal colonic crypts. When viewed microscopically from the luminal surface, ACF have a slit-like appearance with abnormally shaped lumina that are typically larger in size and have a greater space surrounding the abnormal crypt than

normal crypts [52, 59, 60]. Although this is the typical appearance of ACF, there are also histological and dysplastic ACF. Histologically, ACF have larger crypts and wider lumens compared to surrounding crypts. ACF with dysplasia, a hallmark of malignant potential, include crypts with depolarized and stratified nuclei and loss of goblet cells and subsequent loss of mucin [57].

There is currently a concentrated effort to use and verify ACF as a biomarker for colon carcinogenesis. The major advantage to the use of ACF is incidence occurs approximately 30 days or less after carcinogen administration [54]. The early onset of these lesions facilitates the use of short-term assays in animal models. The continued use of ACF have identified that ACF were most commonly found in the distal regions of the colon, with the number of ACF decreasing as it reaches the proximal end of the colon [55]. ACF are most often carcinogen-induced but can also be induced by sporadic mutation.

In a study looking at ACF incidence in different mouse strains, AOM induced ACF in both AOM susceptible and resistant strains. A general trend in ACF frequency 4 weeks after AOM treatment showed that ACF numbers decreased but were restored to higher numbers towards to end of the study. This suggests that ACF are dynamic and endure regression and rebuilding after exposure to chemical carcinogens [14]. Other studies have concluded that ACF induction may be under a different set of genetic controls than tumorigenesis [61] while other studies report an absence in correlation between ACF and tumor development [67-69].

Many studies have been conducted to look at some varying factors of ACF incidence such as carcinogen and its dose, animal species and strain, and administration method and frequency. The most common carcinogens used are azoxymethane (AOM), 1,2 dimethylhydrazine (DMH), and dextran sulfate sodium (DSS) with DSS often occurring in combination with either AOM or DMH. The doses associated with these carcinogens have varied widely across colonic preneoplastic lesion studies in animal models. The rationale for this discrepancy can be found in the way that chemical carcinogens are administered, usually intraperitoneally or subcutaneously. Different strains and species of animals used as models can also affect the ACF outcome as specific animals or specific strains can be more susceptible to carcinogens than others. For example, the A/J mouse strain is highly susceptible to AOM. One study looked at the incidence of ACF in 3 different strains of mice: A/J (very sensitive to AOM), SWR/J (mildly sensitive), and AKR/J (resistant). AOM induced ACF in all strains with A/J strain having a significant number ACF more than SWR/J and AKR/J strains [14]. These factors affecting ACF incidence need to be addressed for each experimental design.

Even though ACF are still considered the “gold standard” of colon carcinogenesis biomarkers, there is growing controversy on the reliability of ACF as a true biomarker for CRC [54]. The ability of ACF to progress towards malignant cancers has been questioned mainly due to the heterogeneous nature of ACF [53]. Many researchers have found that the relationship between ACF and tumor formation is not clear [52, 53, 57, 61, 67-69] and therefore, additional biomarkers are needed. McKinley et al. 2010 note a few issues with the current approach to ACF analysis: 1) Additional steps are required to remove tissue

from fixative solutions, 2) Difficult to obtain high quality images from free-floating samples, 3) Fatigue and error of observer, and 4) lack of consistent sample orientation (proximal versus distal) [55, 57, 64-66]. There is also an issue with the idea that ACF are preneoplastic and their use in short-term assays compared to tumors being the endpoint of long-term assays. The different uses for these endpoints may contradict each other. One study mentions their work with cholic acid in both short and long term assays with ACF and tumors as endpoints in rodents. In the short term assay, cholic acid added into the diet decrease the number of AOM-induced ACF. The long term assay showed the cholic acid promotes development of tumors in the colon. This study suggests that ACF and tumorigenesis are not related [52]. Another experiment conducted by the same group used genistein instead of cholic acid which was used as a chemopreventative agent against CRC. The short term assay found genistein inhibited development of AOM-induced ACF while long term assays saw an increase in colonic tumors with the same dose of genistein [52]. These studies help to convey the idea that ACF and tumorigenesis may not be related and may possibly be controlled differently in the genetic pathways. A few researchers have even found that, even though there are many ACF induced by chemical carcinogens, a small proportion of the ACF numbers actually progress towards tumors [14, 15, 62, 63]. Another source of controversy with ACF studies is that most are short term studies which may be missing the effect of chemical carcinogens during the important promotion and progression stages of carcinogenesis since the studies are often completed before these stages [52]. While ACF studies are advantageous because of the early onset of the preneoplastic lesions in short term assays, the actual ACF assay may not

be as powerful as originally anticipated and may in fact be misleading in evaluating effects of various agents [14,52]. Some researchers, however, believe that ACF may still be used as a biomarker for colon carcinogenesis as long as its usage and parameters are explicitly and completely defined.

ACF were one of the first preneoplastic lesions to be characterized as a potential early biomarker for colon carcinogenesis [59]. While evidence accumulates in favor of other, more defined preneoplastic lesions, it is fortuitous that ACF are available for initial observation of potential carcinogenesis in the colon where cancers in other tissues are not as fortunate. However, additional colonic preneoplasia biomarkers are recommended to study to better understand the relationship between early preneoplasia induction and tumor development. This advance would allow researchers to reach more relevant conclusions considering colon carcinogenesis.

A few of our early studies had used ACF as a primary biomarker for colon carcinogenesis based on the literature available. Results from these studies and additional literature research have demonstrated that a different preneoplastic lesion with a better defined relationship with tumorigenesis is needed. ACF are still counted and reported to contribute to the controversy surrounding the reliability of these lesions as primary biomarkers for colon carcinogenesis.

Mucin-depleted foci (MDF)

Another preneoplastic lesion that is often investigated in addition to ACF is mucin-depleted foci (MDF). The major characteristic of this lesion is the lack of mucin in the

colonic crypts. Mucins are glycosylated proteins that function as a lubricant and protector of the epithelium in the intestines [70]. The crypts involved in MDF have larger and slightly distorted lumen compared to the surrounding crypts [69]. The foci multiplicity of the MDF is a common characteristic that needs to be recognized when analyzing colon tissues for this lesion. Like ACF, the highest frequency of MDF is found in the distal region of the colon [55]. Artifacts are common in colonic tissue that can be caused by damage by instruments or gut-associated lymphoid tissue (GALT) which can also be devoid of HID-AB stain causing GALT to be mistaken for MDF [55]. To differentiate MDF from other artifacts in the tissue, the foci needs to be at least 4 crypts in size and void of alcian blue stain which stains mucin in the crypts. The alcian blue-neutral red staining method is a common approach to MDF identification and the neutral red acts as a counterstain to the alcian blue. While MDF are visible with this method, other research groups have chosen to use a high-iron diamine alcian blue (HID-AB) stain procedure that also stains based on mucin specificity but may be potentially toxic due to the high iron content[53-55,57].

MDF have so far only been identified in rodents, but Femia et al. 2008 set out to find a MDF-like lesion in humans. Human subjects with either family history of FAP or sporadic CRC patients with no family history of CRC were recruited. MDF-like lesions were observed in the two FAP patients and only one MDF-like lesion was observed among all 19 sporadic CRC patients. The average multiplicity of the MDF-like lesions was 33.4 crypts per focus with the range encompassing 3-110 crypts per focus. These MDF-like lesions had varying levels of dysplasia which may represent the very early stages of CRC in humans. It is also speculated that the MDF observed in the FAP patients may be only part

of the total dysplasia found in the lesion area and could in fact overlap with an HID-AB observed ACF. The relationship between MDF in rodents and humans is unknown, but a rat study suggested the MDF were closer to cancer in terms of genetic and molecular alteration than ACF [53, 54, 69]. MDF share similar molecular alterations as other advanced lesions, particularly mutation in the APC gene, a tumor suppressor. The beta-catenin protein is controlled by this gene whose mutated function in the Wnt pathway elicits and increase in uncontrolled cell growth to a cancerous state [53]. The APC mutation in MDF is observed at a similar frequency as seen in tumors, emphasizing the possibility that MDF have a stronger case as a preneoplastic lesion that reliably progresses toward a tumor growth [53].

The main structural characteristic of ACF is an abnormal crypt morphology and larger lumen and the main characteristic of MDF is the lack of mucin. It has been proposed that these two preneoplastic lesions are closely related [53-58]. A number of studies have touched on this subject. One study found that some of the MDF that were observed under a HID-AB stain were dysplastic ACF with defective production of mucin [53]. When analyzing beta-catenin expression in preneoplastic lesions, one study observed significant overlap in the expression between ACF and MDF [56]. The most comprehensive look at the relatedness of MDF to ACF was conducted by Femia et al 2008 which hypothesized that since ACF and MDF had to undergo similar events, like dysplasia and Wnt activation, these lesions could be related. Min mice and F344 rats were given AOM and DMH, respectively to induce preneoplasia. The study stained colon tissues using methylene blue for ACF staining and HID-AB for MDF. The location of each lesion were

mapped and compared to determine a percentage of colocalization. This study found that nearly 50% colocalization was found between ACF and MDF in both mice and rats, which suggests that MDF and ACF could be related preneoplastic lesions [58].

MDF are a relatively new lesion of interest whose relationship with tumorigenesis seems more straightforward. One study notes that MDF are less heterogeneous than ACF which may lead to less scrutiny of MDF as a reliable preneoplastic lesion of colon carcinogenesis [54]. Our previous studies have not confirmed MDF as a biomarker for tumorigenesis. Since a clear relationship is not yet established, extensive testing is imperative to determine the reliability of MDF to convey tumorigenesis potential in carcinogen induced animal subjects.

Beta-catenin accumulating crypts (BCAC)

Beta-catenin is a protein that is involved in the Wnt signaling pathway which plays a pivotal role in colon carcinogenesis [52]. Accumulation of beta-catenin causes dysregulation of the Wnt pathway, allowing for uncontrolled cell proliferation, a key characteristic of carcinogenesis. BCAC is considered a preneoplastic lesion but does not comprise of a physical morphological deformity like the altered crypt shape of ACF, but rather an accumulation of the beta-catenin protein caused by an alteration in the Wnt pathway. BCAC are found in crypts of colons of rodents exposed to chemical carcinogens. The accumulation of beta-catenin reveals the altered Wnt pathway and APC gene indicating the significant role that this preneoplastic lesion plays in colon carcinogenesis [52].

Unlike ACF and MDF, BCAC cannot be detected with general alcian blue or HID-AB staining methods. BCAC requires immunohistochemistry and incubation with a beta-catenin antibody and are more difficult to detect in topographical unsectioned colon samples [54]. Although independent from ACF and MDF, BCAC share a few characteristics with the other preneoplastic biomarkers. BCAC will generally occur in areas that are low in mucin production [54]. Dysplastic areas of the colon are hot spots for BCAC as well as ACF lesions [57].

Beta catenin accumulating lesions are also relatively new to CRC studies. Even though BCAC are an integral part of a known pathway involved in colon carcinogenesis, numerous studies are being conducted to determine the reliability of BCAC as a biomarker for CRC research. BCAC have been detected as early as 5 weeks after AOM exposure, providing evidence that BCAC could be used as a short-term biomarker [52]. A number of studies have been conducted to determine the effects of chemical carcinogens on BCAC incidence. One study observed cholic acid effects on the incidence of preneoplasia in rodents and cholic acid promoted BCAC incidence in the colon mucosa [52]. This study also implicated a high fat diet as colon tumor promoter and BCAC inducer when exposed to AOM. Another study found that beta-catenin gene mutations were frequent and a crucial initiating step for carcinogenesis [56, 57]. Beta catenin-mutations were also present only in dysplastic ACF suggesting that BCAC occurs where there is dysplasia [57]. Other lab groups have researched BCAC as a reliable early-stage CRC biomarker. Our lab group has joined in the effort of determining the effectiveness of BCAC in a mouse model induced with AOM. The results are hypothesized to mimic results

observed throughout the literature of BCAC while providing valuable information about resistant starch preventing additional preneoplastic lesions besides the extensively studied ACF and MDF.

Genetics of CRC

Cancer is a genetic disease, either inherited or induced through mutations in genes. Most CRC cases are classified as sporadic incidence indicating mutations independent of hereditary influence are present. In colorectal tumorigenesis, a genetic mutation induces a precancerous state involving preneoplastic lesions such as ACF and MDF. Subsequent aberrant proliferation of mutated cells and further mutations result in the production of malignant tumors [56, 70]. Tumorigenesis in the colon occurs mainly through inactivation of tumor suppressor genes and activation of particular oncogenes [76, 79]. Other events that are also important to tumorigenesis include mismatch repair and microsatellite instability which affect stability genes in the colon.

Mismatch repair (MMR) corrects small mismatches, insertions, or deletions that spontaneously occur during the DNA replication process. Three proteins (MutS, MutH, and MutL) are responsible for forming the complex that identifies and binds to the error, excises it, and correctly repairs the mismatch. Abnormal or loss of properly functioning MMR system has been implicated in a number of sporadic and inherited cancers [72]. Impaired MMR function can be a result of a mutation in the system or a mutation that causes underproduction of repairs. Additionally, cells with abnormal MMR tend to accumulate errors rather than correcting the errors [71]. Gene sequences are therefore not corrected or preserved and microsatellite fragments are formed.

Microsatellite instability (MSI) is a state of hypermutability that results from an abnormal functioning MMR system. Microsatellites are short, repeated sequences of base pairs that can cause instability in the genome and shorten or lengthen depending of the functional capability of the MMR system [73]. MSI is phenotypic evidence the MMR function is impaired [74]. Hereditary Nonpolyposis colon cancer (HNPCC) is a type of familial genetic induced CRC that involves an inherited mutation in an MMR gene that causes microsatellite repeat sequence errors to remain [71]. HNPCC is specifically termed Lynch Syndrome when there is a known MMR defect [75].

Inactivation of tumor suppression genes also plays an important role in colon tumorigenesis. The p53 gene plays a major role in tumor suppression and regulates important processes such as apoptosis. p53 is the most commonly mutated gene found in tumors and nearly 50% of human tumors have a mutated, deleted, or inactivated p53 gene [76, 77]. While this defect is common to nearly all cancers, it is estimated that greater than 98% of p53 mutations occur by a somatic mutation rather than familial inheritance [78]. While p53 plays a role in all human cancers, the location and type of mutation is unique to the tissue or organ.

The p53 is a general tumor suppressor and can be found in many different tissue sites. The adenomatous polyposis coli (APC) gene is also tumor suppressor gene but has been associated with CRC tumorigenesis [101]. The APC gene acts by preventing uncontrolled cell growth in the colon and rectum and regulating the cell cycle. Mutations in the APC gene cause an inactivation of the gene and can arise through an inherited or sporadic mutation. Beta-catenin is a protein that is important in cellular adhesion along

the Wnt pathway. Mutations in APC and beta-catenin are important for the early stages of tumorigenesis but do require additional mutations to produce cancers [79]. Normal APC function down regulates beta-catenin to keep this protein present in small yet sufficient amounts. When APC is inactivated, beta-catenin accumulates and numerous studies have reported this increase to be associated with colon carcinogenesis [79-82].

Oncogene activation can also influence tumorigenesis. A mutation in the ras gene (K ras) is a point mutation that is responsible for later tumorigenesis stages, particularly the change from adenoma to carcinoma, and is present in nearly 30% of all human cancers [83, 84]. The K ras mutation is found in approximately 32-57% of colorectal cases [85]. In CRC, K ras mutations induce other deletion mutations that target the transforming growth factor beta (TGF-beta) signaling pathway. With the loss of APC and beta-catenin function and the altered TGF-beta pathways, studies suggest that K ras mutations are linked with common CRC pathways [86, 87].

Gut Microbes

Bacteria present in the human gastrointestinal tract are essential to the health of the human host. In mammals, the population of bacteria present is approximately 10^{12} bacterial cells per gram of colonic content and make up roughly 60% of the total fecal weight [88]. These bacteria can be beneficial or pathogenic. Probiotics refer to the beneficial, nonpathogenic organisms that exert an advantageous effect on the host's health. Most probiotics are anaerobes such as bifidobacteria and lactobacillus, which have been implicated in preventing colonic tumorigenesis [89].

A crucial function of gut bacteria is fermentation of non-digestible dietary components and production of energy for themselves as well as the host. Resistant starch, a type of non-digestible carbohydrate, is used as a fermentation substrate that produces short chain fatty acids (SCFA). Normal sources for SCFA include insoluble plant polysaccharides, also known as dietary fiber [90]. The three main SCFA produced by bacterial fermentation are acetate, propionate, and butyrate. While all three have benefits, butyrate is thought to have the most protective effect against CRC. Butyrate inhibits proliferation and induces cancer cell differentiation in animal models as well as human colon carcinoma cell line LIM1215 [91]. In a rat study using low amylose, high amylose, and butyrylated starch based diets, butyrate concentration in the distal colon region was highest in the butyrylated starch diet. The overall tumor incidence in the distal colon was also the lowest with the same butyrylated starch diet. These results are logical because tumor incidence is located in the distal colonic region and higher butyrate concentration was associated with lower tumor incidence [92].

The composition of the gut microbiota, and resulting amounts of SCFA, can change based on geographical location, BMI, and antibiotic use. One study compared the microbiota populations of European and rural African children. Based on the difference in diets, with the rural African diet having a much higher fiber content, huge shifts in bacterial populations were noted between these two dietary and geographical groups. The children from rural Africa had a much higher percentage of the *Bacteroides* species than European children (73% and 27%, respectively) while experiencing a much lower percentage of *Firmicutes* species (12% and 51%, respectively). The greater percentage of

Bacteroides species and number of SCFA found in rural African children could indicate a higher level of potential protective anti-inflammatory effects in the colon [93]. Changes in the microbiota associated with higher BMI values affects the microbial populations by skewing the nutritional balance and therefore the available fermentable substrates to produce beneficial levels of SCFA for decreased CRC risk [94]. Antibiotics are a relatively new influence on the gut microbiota compared to total microbial evolutionary history. The continued use of antibiotics may alter the normal interactions between microbes and host to the degree that autoimmune disorders are increasing in medically developed countries [95].

Analysis completed by our collaborators found that diet changes may also affect microbial populations. Birt and Phillips 2013 found that the phylum level distribution of gut bacteria in rats changed when fed various resistant starches, particularly a native high amylose cornstarch and a high-amylose-lipid complexed starch, when compared to a control corn starch. Three major phyla were observed: *Bacteroides*, *Firmicutes*, and *Actinobacteria*. The HA7-SA diet had the greatest percentage of *Bacteroides* compared to the HA7 and CS diets (68.2%, 46.3% and 3.7%, respectively). The HA7-SA diet also had the smallest percentage of *Firmicutes* and *Actinobacteria* species (27.2% and 1.4%) present compared to the CS and HA7 diets. This study suggests that small changes in the diet can produce great changes in the microbial diversity of the gut [102].

Our studies have looked at the effect of antibiotics as an additional pressure on the gut microbiota. Our results suggested that using a mixture of broad and specific antibiotics placed a great stress on the microbiota. It is hypothesized that inhibition of

preneoplastic lesions, like ACF and MDF, occurs as resistant starch undergoes greater fermentation than normal starches and therefore greater amounts of SCFA are produced. The greater amount of fermentation products implicates the microbial population is an important factor in CRC risk.

Overall Summary

Many studies have advocated the benefits of RS in colon health. The aim of this study was to determine the potential health benefits of four different RS on the inhibition of colon carcinogenesis biomarkers. The four RS used in these studies varied in resistant starch content and structure. Both mouse and rat models were used to try to reproduce results in two separate species thus giving the results added support for RS inhibition of preneoplastic lesions. While ACF and MDF have both been extensively studied, a new biomarker with a better known mechanism would allow for the next step of RS utilization to be used for human subjects.

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CHAPTER 3: EFFECT OF DIETARY RESISTANT STARCH ON INHIBITION OF PRENEOPLASIA IN AZOXYMETHANE-INDUCED RODENT MODELS

Abstract

Numerous studies suggest that dietary fiber could improve colon health and prevent incidence of preneoplastic colon lesions. Preneoplastic lesions, such as aberrant crypt foci (ACF) and mucin-depleted foci (MDF), have been extensively studied as potential biomarkers for colorectal carcinogenesis. The aim of this study was to determine the possible inhibitory effect of resistant starches, a novel type of dietary fiber, on the development of colonic preneoplastic lesions in azoxymethane (AOM) treated rat and murine models fed resistant starches at 55% of the diet beginning after AOM treatment and for 10 weeks. Another objective was to determine what effect resistant starches would have on the development of preneoplasia when the animals were subjected to an antibiotic (Ab) treatment following azoxymethane and before resistant starch diets were fed. The antibiotic treatment consisted of a three day administration of 50mg/ml vancomycin and 50mg/ml imipenem via drinking water. Three resistant starches, high-amylose (HA7), high-amylose-octenyl succinic anhydride (OS-HA7), and high-amylose-stearic acid (SA-HA7) were compared against a control commercially available corn starch (CS). The animals treated with AOM and fed the SA-HA7 diet, despite the high resistant content, consistently developed the highest average ACF per animal. In the rat study, there were significant differences in ACF numbers between the AOM treated rats fed the HA7 and OS-HA7 diets ($p=0.04$) and between AOM treated-antibiotic administered rats fed the CS and SA-HA7 diets ($p=0.008$). In the mouse study, a

significant difference in ACF numbers was noted between the AOM treated HA7 and SA-HA7 fed mice ($p=0.04$). There were no MDFs observed in the colons of the mice or the rats fed the SA-HA7 diet in both AOM only and AOM treated-antibiotic administered rats. For antibiotic treated rats, MDF were only observed in rats fed the CS diet. MDF were unexpectedly present in CS fed rats treated with saline and antibiotics. Further long-term studies need to be conducted to determine if resistant starch impacts colon carcinogenesis in rodents.

Introduction

Colorectal cancer (CRC) is the third most prevalent cancer in both men and women in Western countries and the second leading cause of cancer deaths. The American Cancer Society predicts nearly 103,000 new cases in 2013 [1]. Most of these new cases are classified as sporadic, compared to hereditary types of CRC, and are greatly influenced by epigenetic factors such as diet and lifestyle.

The human diet, on average, consists of approximately 55% starches and other carbohydrates [14]. Dietary fiber refers to indigestible carbohydrate portions of plant products that are made of soluble and insoluble fibers. Both components offer beneficial effects on the colon but the insoluble portion is of interest in this study. The insoluble parts of dietary fiber provide beneficial health effects such as blood sugar regulation, fecal bulking and overall colon health [2]. Some previous studies provide evidence that consumption of dietary fiber or other slowly digestible starches reduce overall CRC risk as well as reduce the number of colonic preneoplasia, aberrant crypt foci (ACF) and mucin-depleted foci (MDF), and adenocarcinomas [3-6].

Resistant starch (RS) has been considered a third type of fiber that has the beneficial effects of both the soluble and insoluble components. RS is the portion of starch that escapes normal digestion in the small intestine and instead passes on to the large intestine. The component that enters the large intestine is referred to as the resistant starch content [15]. There are four classified types of resistant starches. Type 1 RS is physically inaccessible starch that is often found in unprocessed whole grains, seed and legumes. Type 2 RS is a naturally occurring crystalline starch structures commonly found in uncooked potatoes and high-amylose starches. Type 3 RS is created when food are cooked and cooled and can be found in foods like bread, pasta, and potatoes. Type 4 RS does not occur naturally as it is a chemically modified starch. A new type of RS, type 5 RS has been developed that complexes a high amylose starch with a stearic acid lipid (SA-HA7). The effects of this novel starch on the inhibition of colonic preneoplasia were analyzed in this study.

The objective of this research was to determine the effects of different resistant starches on the inhibition of colonic preneoplastic lesions ACF and MDF. It is hypothesized that the novel high-amylose-lipid complexed starch (SA-HA7) will have the greatest inhibition on preneoplastic lesions in azoxymethane (AOM) treated animals when compared to the HA7 and OS-HA7 diets, due to the high percentage of indigestible components that enter the colon. Azoxymethane (AOM) is commonly used in animal models to induce colon carcinogenesis and is frequently used instead of its metabolic precursor 1,2-dimethylhydrazine (DMH) owing to the increased potency and reduced inflammation with AOM compared to DMH [7,8]. The overarching aim is to find a RS that

shows the greatest potential for inhibiting colonic preneoplasia to eventually be put into a food product. The results from this study, and similar studies could have a lasting impact on the prevention on colon carcinogenesis by means of a resistant starch replacing normal starches in processed foods for human consumption.

Materials and Methods

Animals and husbandry

Ninety 5-week old male F344 rats and 120 5-week old male A/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were housed individually. Animals were placed on wire floors for the duration of the experimental feeding but not during the times of acclimation or injections. The animals were kept in controlled rooms at $20\pm 3^{\circ}\text{C}$, $50\pm 5\%$ humidity, and a 12 hour light/dark cycle. Food and water was administered *ad libitum*. Body weights were recorded weekly. All animal care, handling, and procedures were approved by the Iowa State University Institutional Animal Use and Care Committee and conducted in accordance with the Institutional Animal Care and Use Committee guidelines.

Starch processing, materials, and resistant starch content analysis

Four starches were evaluated in these experiments: Control cornstarch (CS), high amylose cornstarch (HA7), high amylose-octenyl succinic anhydride modified cornstarch (OS-HA7), and a novel resistant starch high amylose-stearic acid complexed cornstarch (SA-HA7). CS and HA7 were purchased from Oziama Inc. (Cargill distributor, St. Paul, MN) and stearic acid and octenyl succinic anhydride were purchased from Sigma-Aldrich (St.

Louis, MO). The OS-HA7 starch was processed according to Zhang et al. 2011 while the SA-HA7 starch was processed according to Hasjim et al. 2010 [9,10]. Resistant starch content, measured in % resistant starch, was determined using AOAC 991.43 and Englyst [18] methods.

Diets and preparation

The diets were based on the AIN-93 laboratory animal diet (Harlan Teklad Animal Diets, Madison, WI) with the difference between the diets occurring in the type of corn starch used (Table 1). For each diet, starch comprised of 55% of the total diet. The starches were cooked using a water-boiling method with a starch-to-water ratio of 1:3 and were prepared and administered according to Zhao et al. 2011 [2].

Rat experimental feeding procedure

The rat experiment had two primary objectives. The first objective, the diet objective, analyzed the effect of RS diets on preneoplasia inhibition with the use of four RS diets: CS, HA7, OS-HA7, and SA-HA7. The second objective, the antibiotic objective, analyzed the effect of antibiotic administration on preneoplasia inhibition using only the CS and SA-HA7 diets. Animals in both objectives were subjected to a carcinogen or saline treatment (s-saline, a-AOM). The mice in CS-s, CS-a, SA-HA7-s, and SA-HA7-a groups in the diet objective were used as controls for comparison in the antibiotic objective.

Upon arrival, all rats were fed the control starch diet for a one week acclimation period. After acclimation, the rats were divided into 12 treatment groups, six of which received weekly AOM dose of 15mg/kg body weight AOM by intraperitoneal injection

(i.p.) for two weeks (10 animals per treatment group). The other six treatment groups were i.p. injected with equivalent volumes of saline to act as a control treatment (five animals per treatment group). Body weights were recorded for three days following each injection. All animals were fed the CS diet during AOM/saline treatments. Starting three days following the last AOM or saline injection, the animals were fed the experimental diets CS, HA7, OS-HA7, and SA-HA7 (15 animals per diet group-10 for AOM and five for saline treated groups). Four of the treatment groups were given an antibiotic mixture of 50mg/ml vancomycin and 50mg/ml imipenem (Lloyd Veterinary Medical Center, Iowa State University, Ames, IA) administered via drinking water for three days starting the day after the second AOM/saline injection. This antibiotic mixture and administration protocol was based on Manichanh et al. 2010 [17]. All groups were fed the experimental diets for 10 weeks. The rats were sacrificed using CO₂ euthanasia. The distal 5cm of the colon was excised, cut open longitudinally, flattened between two slides, and placed in 10% neutral buffered formalin for tissue analysis.

Mice experimental feeding procedure

The same four diets (CS, HA7, OS-HA7, and SA-HA7) were used for the mice feeding experiment. The mice were placed on the CS diet during an initial two week acclimation period. After acclimation, the mice were divided into eight treatment groups (four diets, each with an AOM and saline treatment). The AOM treated animals (10 animals per group) were given a dose of 7.5mg/kg body weight by i.p. injection weekly for four weeks. Saline treated animals (five animals per group) were given equivalent

volumes as a control weekly for four weeks. Three days following the last injection, all mice were placed on wire floors. A prior study recommended a 10mg AOM/kg body weight dose for the same age and strain of mouse [16]. Subsequent studies in our lab suggested 10mg AOM/kg body weight administered to mice living on wire racks induced death by liver toxicity and found that a dose of 7.5mg AOM/kg body weight balanced toxicity with sufficient induction of preneoplastic lesions. Body weights were recorded for three days following each injection treatment. All animals were fed the CS diet during AOM/saline treatments. Starting three days after the last AOM injection, the mice were fed the experimental diets for 10 weeks. Mice were sacrificed using CO₂ euthanasia. The entire colon was removed, cut open longitudinally, flattened, and placed in 10% neutral buffered formalin for tissue analysis.

Analysis of preneoplastic lesions ACF and MDF

Colon samples were removed from slides and rinsed with distilled water. The colon sample was stained with 1% alcian blue for 6-7 minutes then counter-stained with 1% neutral red for 30 seconds (Sigma-Aldrich, St. Louis, MO). The samples were rinsed with distilled water, placed on a glass slide and examined microscopically for ACF and MDF lesions.

Statistical analysis

Statistical analysis of ACF and MDF of the rat diet objective was conducted using a two-way ANOVA to determine diet and treatments effects, as well as any significant

interactions. A three-way ANOVA was conducted for ACF and MDF in animals on the antibiotic objective of the rat study to determine potential diet, treatment, or antibiotic effects as well as any significant interactions.

Due to the low numbers of ACF observed in mice, a generalized linear model (GLM) was used to determine diet and treatment effects. A GLM procedure was used instead of a two-way ANOVA because of the non-normal distribution of the mouse ACF results. *Post hoc* t-tests were conducted to determine significance of diet and treatment effects between two groups. Statistical significance is determined by a p-value lower than 0.05.

Results

Body weights

Body weights were recorded weekly as well as for three days following each injection of AOM or saline in both rat and mouse studies. The weight gain of the rats was similar for all diets and treatments in both objectives (Figures 1 and 2). Due to the similar weight gain, statistical analysis of the rat body weight data was conducted on the entire experiment duration of data. The weight gain in the rat study was found to not be significantly different between diet, carcinogen, and antibiotic groups. The weight gain difference in the mouse study was more noticeable (Figure 3) and the analysis was separated into two analyses due to the different experimental feeding conditions with the CS diet fed weeks 1-6 and experimental diets fed weeks 6-17. Analysis revealed that during the initial CS-only feeding period, there a significant carcinogen treatment effect (ANOVA $p < 0.0001$). Analysis at week 7, 12, and 17 found that while the OS-HA7-s and OS-

HA7-a groups were not significantly different from each other, the OS-HA7-a diet group was significantly different from all other diets at these times points.

Resistant starch content

The resistant starch contents of the experimental diets are shown in Table 2. Results for both AOAC 991.43 and Englyst methods are presented. In AOAC-analyzed starches, all starch diets contained significantly different percentages of resistant starch content. In the Englyst method, the resistant content of the HA7 and OS-HA7 diets were not significantly different ($p=0.07$) based on our chosen significance level of $p<0.05$.

Preneoplastic lesions

Aberrant crypt foci in the diet objective of the rat study were only observed in the AOM treated rats (Figure 4). A significant difference between saline and AOM treated animals was observed (ANOVA $p<0.01$) but there was no significant difference in the number of ACF between diets of AOM treated animals. The OS-HA7-a and SA-HA7-a groups were not significantly different from each other, but were trending towards the significance cut off (*post hoc* $p=0.07$). SA-HA7 fed-AOM treated rats had the greatest average number of ACF per rat while the fewest average number of ACF were observed in OS-HA7 fed-AOM treated rats, 34.1 ± 32.4 and 17.1 ± 16.2 ACF, respectively. The ACF in HA7 fed-AOM treated rats (29.5 ± 14.9 ACF) were fewer than those found in SA-HA7 fed-AOM treated rats (34.1 ± 32.4 ACF) but greater than the CS fed-AOM treated group (22.7 ± 12.1 ACF).

The antibiotic objective of the rat study also displayed a significant AOM group versus saline group effect on ACF numbers (Figure 5, ANOVA $p < 0.01$). The greatest number of ACF was in the SA-HA7 fed-AOM treated group (34.1 ± 32.4). There was a significant antibiotic effect observed in AOM treated rats within both CS and SA-HA7 diets (ANOVA $p < 0.0001$). ACF in CS-a-Ab and SA-HA7-a-Ab groups were also significantly different (*post hoc* $p = 0.008$).

The average ACF per mouse data can be found in Figure 6. Like the rat studies, the greatest number of ACF were present in the SA-HA7 fed-AOM treated mice (1.7 ± 1.8 ACF per mouse). The mouse study, however, differed from the rat study in which AOM treated experimental diet group had the lowest number of ACF. The HA7-fed-AOM treated mice had an average of 0.6 ± 0.8 ACF per mouse, indicating a greater degree of preneoplasia inhibition. Overall, ANOVA results suggest there was no significant diet effect across all diet groups but the SA-HA7-a and HA7-a groups were significantly different in ACF numbers determined by *post hoc* tests ($p = 0.04$).

ANOVA results also suggested there was no significant difference in MDF numbers in rats in the diet objective (Figure 7). Even though the greatest number of ACF were observed in the SA-HA7-a group, this group also saw zero MDF, in AOM treated rats. MDF were expected to be found only in AOM-treated rats, but were found in saline treated rats in the diet objective only in CS and OS-HA7 diets.

MDF results in the antibiotic objective can be found in Figure 8. Zero MDF were found in all SA-HA7 rats, both AOM and saline treated animals. Due to this lack of MDF in the SA-HA7 fed rats, there is a significant difference between the two diets, CS and SA-

HA7 (ANOVA $p < 0.001$). Within the CS diet, there was no significant difference between AOM groups versus saline groups. The saline and antibiotic treated rats in the CS diet group (CS-s-Ab) developed the greatest number of average MDF per rat (1.8 ± 1.9 MDF) compared to the other CS subgroups but was not significantly different from the CS-a-Ab group (*post hoc* $p = 0.16$). The antibiotic treated groups in the CS diet were significantly different compared to the SA-HA7 diet counterparts (Figure 8). No data is presented for mouse MDF as there were no MDF observed in any mouse from any diet and treatment.

Discussion

Our initial hypothesis stated that the SA-HA7 starch diet would exhibit the greatest inhibition of both ACF and MDF preneoplastic lesions in rat and mouse models. This was based on the rationale of SA-HA7 containing the greatest percentage of resistant starch content, approximately 30-35%, compared to the lower percentages of resistant starch content found in the other three diets (Table 2). The SA-HA7 inhibition hypothesis was also based off of previous studies conducted in the lab where the SA-HA7 diet significantly reduced the number of ACF and MDF in the colons of rats [4]. In the current study, the animals treated with AOM and fed the SA-HA7 diet, in fact, developed the greatest number of ACF in both animal studies, 34.1 ± 32.4 ACF per rat and 1.7 ± 1.8 ACF per mouse. Overall, ACF differences between the diets of the AOM treated animals were not significant (ANOVA, $p > 0.2$) but nearly every aspect of both animal studies observed SA-HA7-fed animals with the greatest number of ACF. The only deviance from this statement can be found in the ACF results in the antibiotic objective of the rat study (Figure 5). The SA-HA7-a-Ab treated rats experienced a significantly greater inhibition of ACF than its CS

diet counterpart ($p=0.008$). This result could implicate a potential synergistic effect between a high resistant starch content starch and antibiotic treatments.

The HA7 and OS-HA7 diets provided the greatest protection against incidence of preneoplastic lesions, although the diet that provided the greatest inhibition of ACF lesions was different between the rat and mouse studies. All ACF results can be found in Figures 4-6. The diet objective of the rat study suggested that the OS-HA7 diet had the greatest preneoplasia inhibition, approximately 40% reduction from the CS diet, while the mouse study determined the HA7 diet to exhibit nearly 50% reduction in average ACF per mouse from the CS-fed mice. These differences, however, are not statistically significant (ANOVA $p>0.2$), but they provide insight to the challenges in researching a complex subject like CRC in highly variable animal models.

Previous studies suggested that MDF may be a better biomarker for colon carcinogenesis than ACF [11-13]. One study noted that MDF may be subjected to less scrutiny than ACF because MDF are less heterogeneous than ACF [12] and the APC mutation associated with CRC is observed in MDF and tumors in similar frequencies [11]. MDF results for the rat objectives are presented in Figures 7 and 8. MDF were not observed during tissue analysis in the mice tested in this study. The SA-HA7 diet in both rat objectives inhibited the production of MDF in the distal colon compared to the other diets and treatments. MDF were observed in similar frequency in HA7 and OS-HA7 fed rats treated with AOM. Saline treated, CS-fed and OS-HA7-fed rats were the only groups that developed MDF.

Possibly the most surprising results came from the MDF data of the antibiotic treated rats. The greatest number of MDF for this objective in the rats was found in the CS-s-Ab treated rats with approximately 1.8 MDF per mouse. The next highest number of MDF was found in the CS-a-Ab treated rats, an average of 0.8 MDF per rat. The high numbers of MDF found in saline treated animals calls into question the relevance of MDF as a biomarker for CRC since there is no evidence that antibiotic treatments would induce CRC.

While ANOVA analyses and *post hoc* t-tests determined the numbers of ACF and MDF of most diet and treatment groups were not significant, the trends are still important for interpretation of the results. The original hypothesis of SA-HA7 providing the greatest inhibition of preneoplasia was half supported. The SA-HA7 diet inhibited MDF in both objectives but only significantly in the antibiotic objective (*post hoc* $p=0.011$) but enhanced ACF production in both objectives compared to the CS diet in AOM treated rats. The enhanced ACF production was also observed in mice, significantly different from the diet group with the lowest average of ACF per mouse found in the HA7 fed mice ($p=0.04$). Similar trends were expected across the rat and mouse study results, but this hypothesis was not supported as the experimental diet feeding yielded different results between the two animal studies. The HA7 diet was associated with higher ACF and MDF production in the rats but with lower yields of ACF in mice. The OS-HA7 diet was associated with fewer ACF but more MDF in rats and more ACF in mice.

In conclusion, the animals fed the diet containing starch with the highest resistant starch content percentage, SA-HA7, experienced the induction of the greatest number of

ACFs in AOM treated rodents. Feeding the diet containing the lowest resistant content percentage, CS, resulted in AOM treated animals with high numbers of ACF, while feeding the diets containing middle-range resistant starch content, HA7 and OS-HA7, saw inhibitory effects of ACF in AOM treated rodents. This suggests that there may be a dose-response occurring with starch resistant content and ACF incidence. The MDF results suggest that MDF may not be a reliable or relevant biomarker for CRC induction. While there is a lot of variability associated with animal studies, additional long term studies need to be conducted to evaluate and compare preneoplasia data with tumor results in rodents treated with carcinogens and fed RS diets.

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DATA

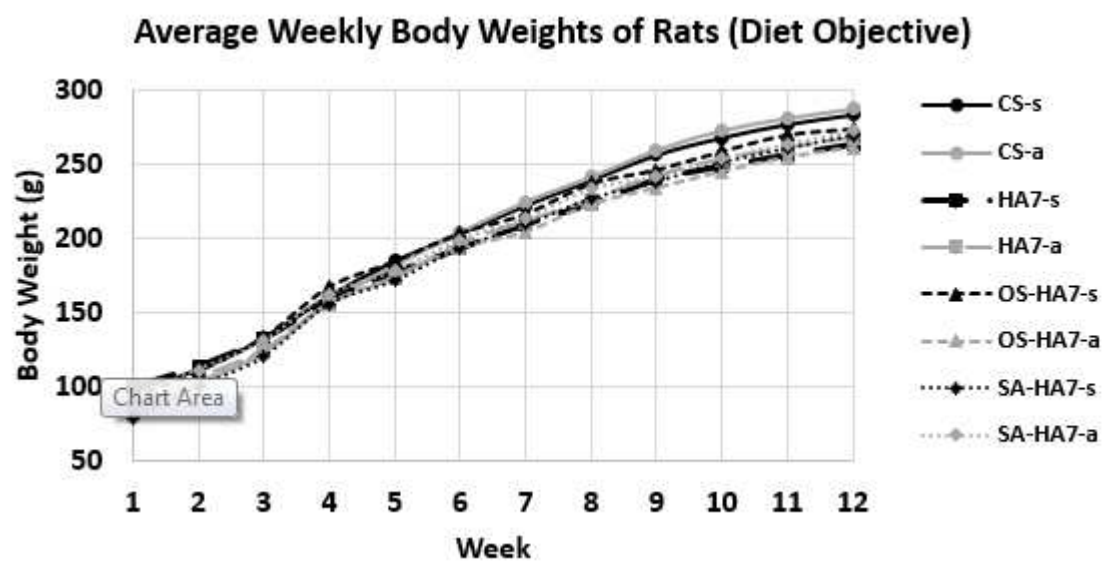


Figure 1: Average weekly body weights in grams (g) of rats on experimental diets. No significant differences observed between diets at any week (ANOVA $p < 0.2$ for diet, treatment, and interaction). Note: s-saline, a-AOM.

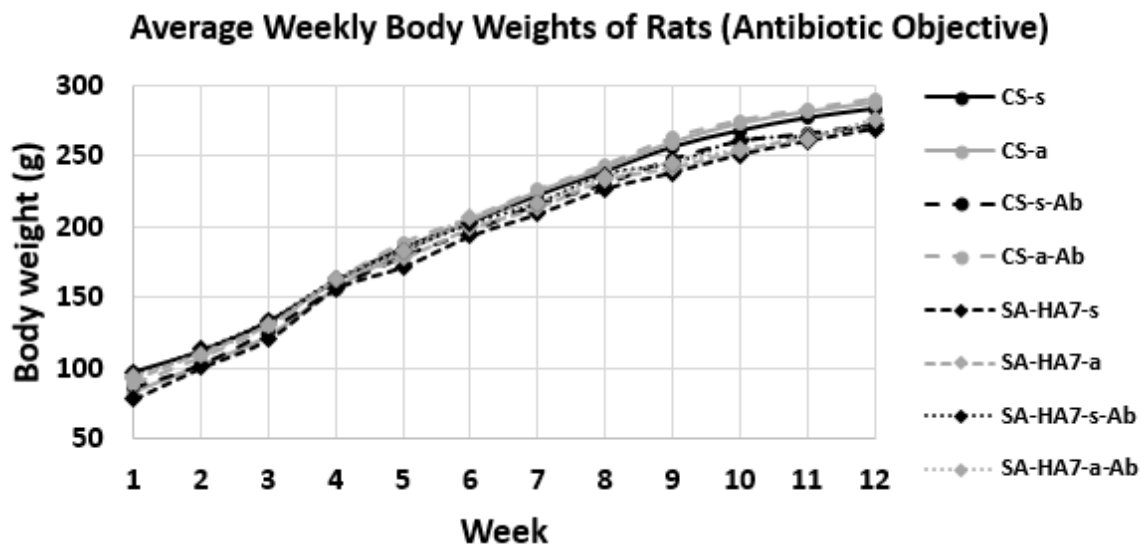


Figure 2: Average weekly body weights in grams (g) of rats on experimental diets with and without antibiotic treatments. No significant differences observed between diets at any week (ANOVA $p > 0.2$ for diet, treatment, and interaction). Note: s-saline, a-AOM, Ab-antibiotic treatment administered.

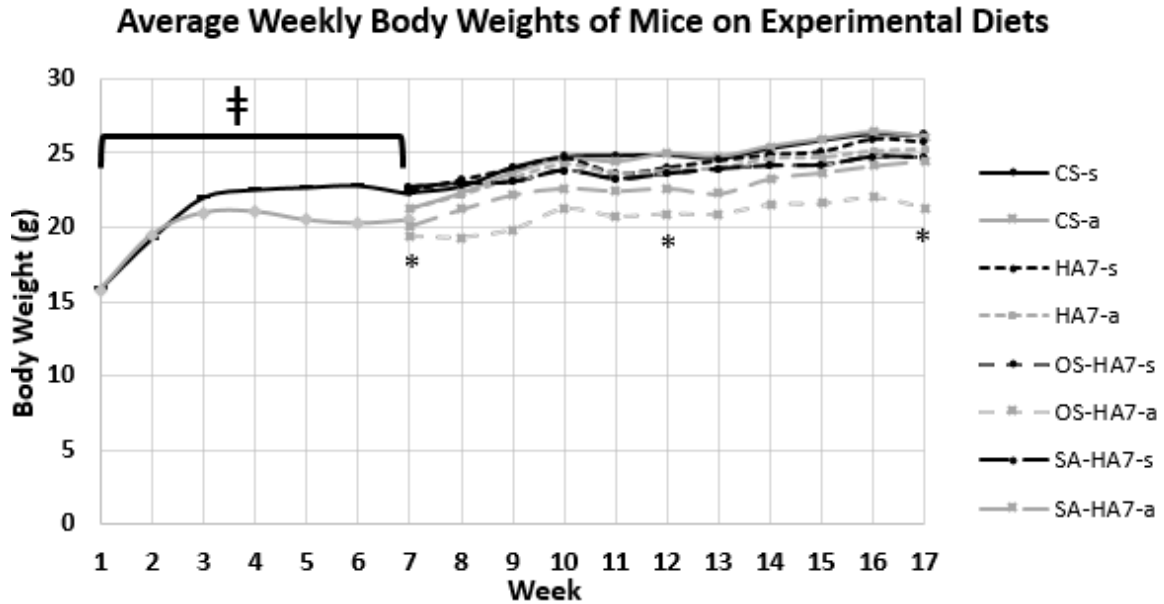


Figure 3: Average weekly body weights in grams (g) of mice on experimental diets and carcinogen treatments. Weeks 1-7 all mice were fed CS diet. Only two lines, CS-s and CS-a are graphed for weeks 1-7 as all animals were fed the CS diet during this time period. ¶ denotes significant ANOVA treatment effect for weeks 1-7 (ANOVA $p < 0.0001$). Weeks 17 denotes weight on day of sacrifice. Weeks 7-17 mice were fed experimental diets and there was a significant diet effect but no significant treatment effect (ANOVA diet $p < 0.0001$, treatment $p > 0.05$). Values denoted with * are significantly different at $p < 0.05$ by *post hoc* tests. Note: s-saline, a-AOM.

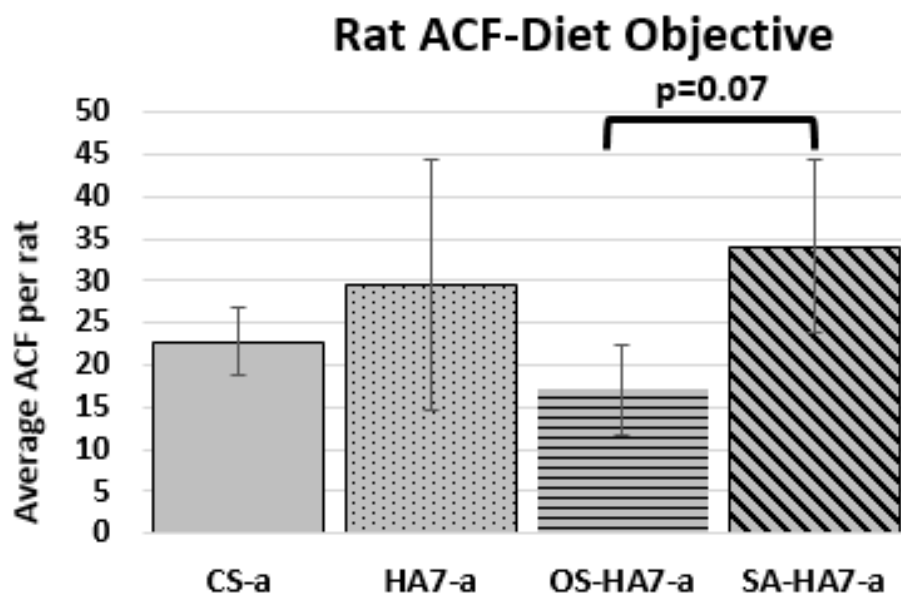


Figure 4: Average ACF per rat in experimental diets objective. Values for saline treated animals not shown since ACF only observed in AOM treated rats. No significant diet or interaction effect was observed, but a treatment effect was observed (ANOVA diet $p>0.05$, interaction $p>0.05$, treatment $p<0.01$) but OS-HA7 compared to SA-HA7 trending towards significance (*post hoc* $p=0.007$). Values shown are mean \pm standard error.

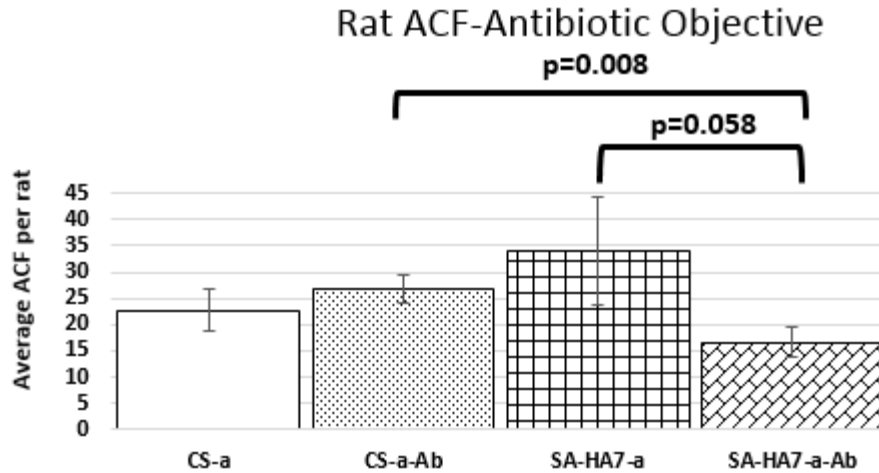


Figure 5: Average ACF per rat in antibiotic treatment objective. Saline treated groups did not produce ACF and are not presented on the graph. No significant diet effect or interaction, but a significant treatment effect was observed (ANOVA diet $p>0.05$, interaction $p>0.05$, treatment $p<0.01$) Significant diet effect observed in AOM and antibiotic treated rats (*post hoc* $p=0.008$). Data presented are mean \pm standard error. Note: a-AOM, Ab-antibiotic treatment administered.

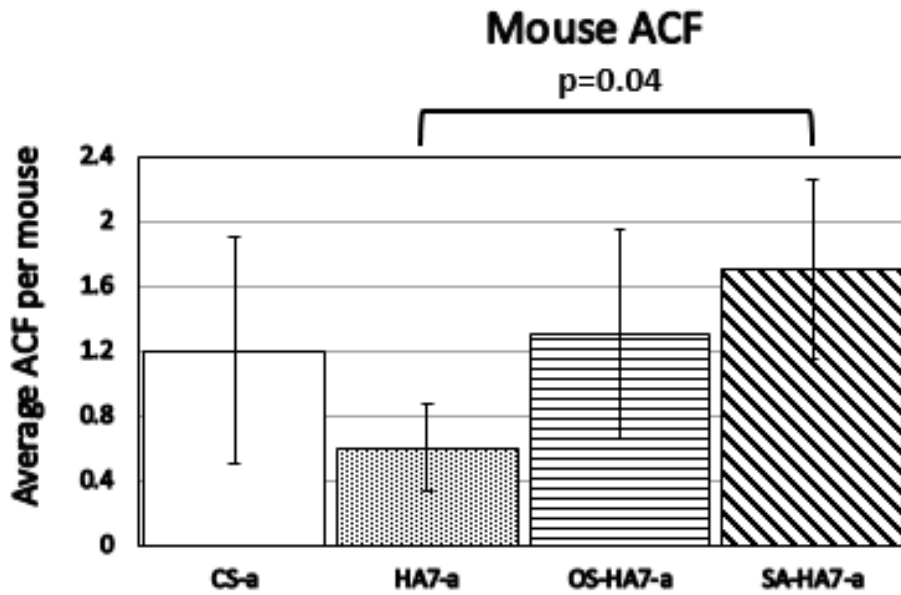


Figure 6: Average ACF per mouse fed experimental diets. Data from saline treated animals not shown as all saline animals did not produce ACF. No overall significant diet effect or interaction observed (ANOVA diet $p>0.2$, interaction $p>0.05$). A significant treatment effect was observed (ANOVA treatment $p<0.03$). Significant difference between HA7 and SA-HA7 diet is noted (*post hoc* $p=0.04$). Data shown represent mean \pm standard error.

Note: a-AOM.

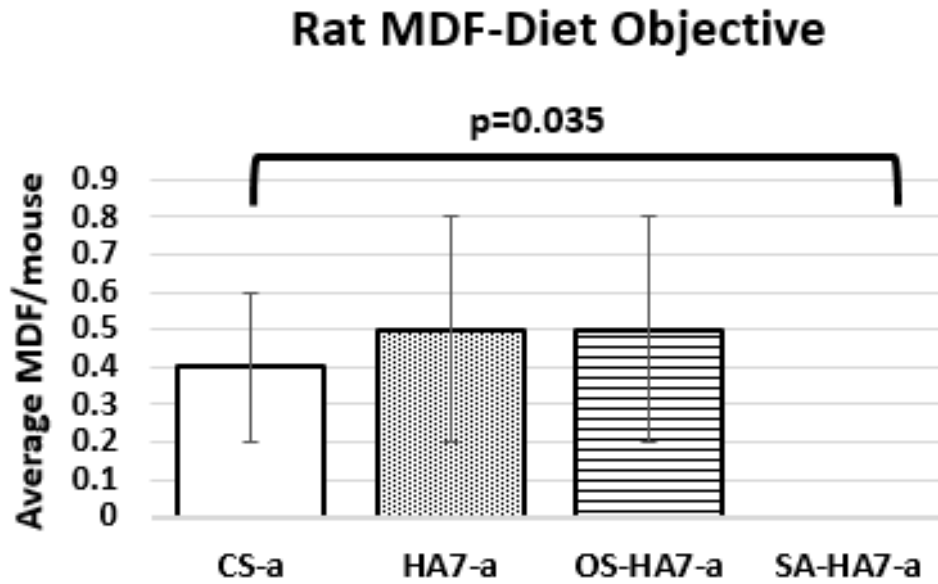


Figure 7: Average MDF per rat in the experimental diets objective. Data from saline treated animals not shown as all saline animals did not produce MDF. No diet or interaction effect was observed but there was a significant treatment effect (ANOVA diet $p>0.05$, interaction $p>0.05$, treatment $p<0.01$). Significant difference between CS and SA-HA7 diets was noted (*post hoc* $p=0.035$). Values shown are mean \pm standard error. Note: a-AOM.

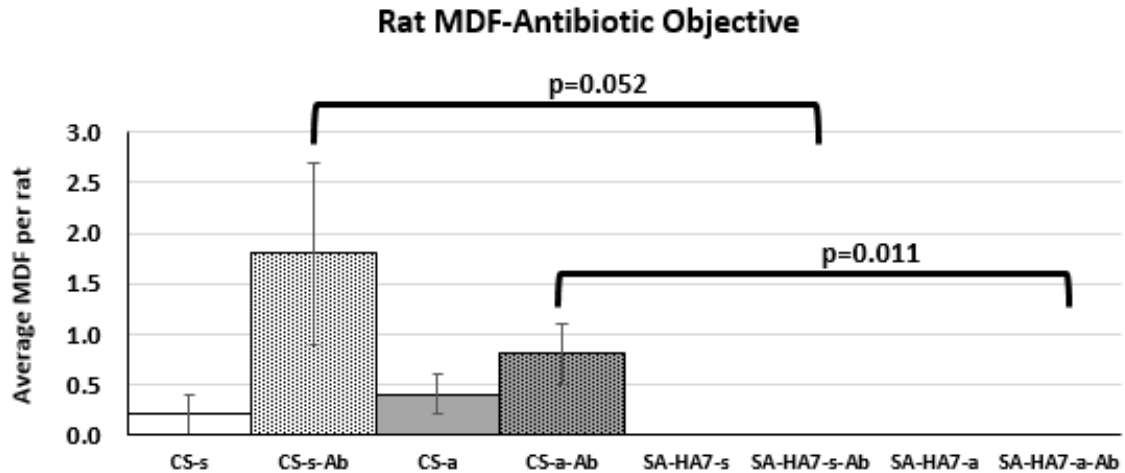


Figure 8: Average MDF per rat in antibiotics objective. Data presented are mean \pm standard error. Note that SA-HA7 diet produced no MDF. ANOVA results indicated that there was a significant diet, antibiotic, and diet*antibiotic interaction effect, and no significant treatment or all other interaction effect (ANOVA diet $p < 0.001$, antibiotic $p < 0.0001$, diet*Ab $p < 0.03$, treatment $p > 0.05$, other interactions $p > 0.05$) Significant diet effects between saline-Ab treated rats and AOM-Ab treated rats (*post hoc* CS-s-Ab vs. SA-HA7-s-Ab $p = 0.052$, CS-a-Ab vs. SA-HA7-a-Ab $p = 0.011$). Note: s-saline, a-AOM, Ab-antibiotics administered.

Table 1: Diet Formulation for Animal Diets^a

Diet Ingredient	Percentage of Total Diet
Starch	55%
Casein	20%
Dextrose	15%
Mineral Mix	3.5%
Vitamin Mix	1.0%
Methionine	0.3%
Choline	0.2%
Corn Oil	5.0%

^a Starches were purchased from Cargill Inc. (Minneapolis, MN) and used as received or furthered process as described in methods section. Non-starch ingredients were purchased from Harlan Tekland Diets (Madison, WI). Diet formulation based on standard AIN-93 lab animal diet formulation. Mineral and vitamin mixes are standard AIN-93 mixes.

Table 2: Resistant starch content of resistant starch diets for both rat and mouse model studies^b

Starch Diet	Resistant Starch Content %	
	AOAC 991.43	Englyst
CS	3.1 ± 0.4 ^a	1.8 ± 0.3 ^a
HA7	16.1 ± 1.8 ^c	11.3 ± 0.9 ^b
OS-HA7	5.7 ± 0.8 ^b	14.3 ± 1.0 ^{bc}
SA-HA7	33.0 ± 1.4 ^d	18.2 ± 0.5 ^d

^b Resistant starch content of diets were measured by AOAC method 991.43 and Englyst methods. Data is presented as mean ± standard error. Values within each analysis method column without a common letter superscript are significantly different at p<0.05.

CHAPTER 4: GENERAL CONCLUSIONS

Resistant starch is a natural component found in many green plants. RS has shown to have a number of beneficial health effects including improved colon health through fecal bulking, increased water absorption, decreased fecal transit time, and fermentation by gut microbiota [1-3]. Resistant starch escapes normal digestion in the small intestine and is instead passed onto the large intestine where it is fermented by the gut microbiota to produce SCFA that have also been associated with decreased CRC risk and increased bowel health [3-5]. It has been classified as a dietary fiber which has been associated with decreased risk of CRC [6].

Our studies tested three different resistant starches for their ability to inhibit induction of colonic preneoplasia in two different animal models. Male F344 rats and A/J mice were fed experimental diets containing normal control cornstarch (CS) or one of the 3 resistant starches: a natural high-amylose cornstarch (HA7), a chemically modified high-amylose octenyl succinate starch (OS-HA7), or a high-amylose-stearic acid complexed starch (SA-HA7). The proportion of starch that reaches the colon to be fermented is referred to as the resistant starch content percentage [7]. The order of increasing resistant starch content found in each starch determined by AOAC 991.43 method is as follows: CS, OS-HA7, HA7, and then SA-HA7 with the greatest resistant content. The Englyst method order of increasing resistant starch content is as follows: CS, HA7, OS-HA7, and then SA-HA7. No significant difference observed between the HA7 and OS-HA7 diets. It was hypothesized that the starch with the greatest resistant content would demonstrate the greatest inhibition of preneoplastic lesions ACF and MDF.

Azoxymethane was used to induce preneoplastic lesions in mice. Results from AOM treated mice were compared to saline treated control animals. ACF and MDF were counted microscopically in the colons of animals 10 weeks after the last carcinogen or saline treatment. Azoxymethane was chosen for our studies because of its greater stability and potency over another common CRC carcinogen, DMH [8]. Azoxymethane induces DNA damage by producing DNA-reactive adducts without additional inflammation and produces sufficient numbers of ACF and MDF for complete analyses [9]. The dose and regimen was different between the rat and mouse studies. A typical dose and regimen for rat CRC studies using AOM is two weekly intraperitoneal injections of 15mgAOM/kg body weight while mice studies typically use 4 injections of 10mgAOM/kg body weight [9-12]. Previous studies in our lab found that the rat dose and regimen was sufficient in producing ACF and MDF in the rat colons. Mice, however, were susceptible to fatal liver toxicity at the 10mgAOM/kg body weight dose after only two of the four total injections and were placed on the wire racks early which may have also contributed to total stress. The results from this previous study led to a dose study to find an appropriate balance between toxicity and induction of sufficient number of lesions for analysis which provided an optimal dose of 7.5mgAOM/kg body weight in mice.

The choice of animal model was important to recapitulate previous preneoplasia induction results. Rodent models are common in biological studies due to similar physiologies with humans as well as a shorter lifespan that allows for fast and reproducible studies. Our studies involved both F344 rats and A/J mice for AOM-induced preneoplasia. Previous studies suggested that rats may be a better model for inducing,

observing, and characterizing MDF since MDF were considered a more reliable and relevant biomarker for CRC [11]. Our data, however, suggested that MDF may not be a reliable biomarker as MDF were observed in the greatest numbers in saline treated animals, particularly with antibiotic treatments. Following the rat study, it was decided to use a mouse model that was susceptible to AOM-induced preneoplasia. Our studies found no significant differences in ACF numbers overall between the four diet groups. Specific comparisons, particularly between the HA7 and SA-HA7 fed and AOM treated mice, were found to be significantly different. Zero MDF were found in the mice.

The two main lesion biomarkers of interest were ACF and MDF. ACF were one of the first preneoplastic lesions to be observed and characterized for CRC [13]. ACF were characterized by an aberrant crypt morphology with a slit-like appearance and increased crypt cell lumen. These characteristics were observed in whole mount colon preparations of both mice and rats 10 weeks after the last carcinogen treatment. In general, the ACF data did not produce statistically significant results but did provide some interesting trends. In both rat and mouse studies, the animals fed the SA-HA7 diet had the greatest number of lesions, although not statistically significant. The only statistically significant difference in ACF data was between AOM treated rats treated with antibiotics and fed CS or SA-HA7 diet. This was also the instance where ACF numbers in the SA-HA7 fed diet were less than CS data. ACF have come under scrutiny as previous studies suggest a small percentage of ACF progressing towards malignant tumors [12, 13]. With the increased scrutiny, it was important to identify another potential biomarker for CRC.

MDF were also analyzed in our animal studies. MDF are characterized by the diminished or lack of mucin production in the crypt cells of the colon which aid in lubrication and protection of intestinal epithelium [15, 16]. When looking at only diet effects on MDF production, there was no significant difference in the average number of MDF between diet groups. There was no AOM versus saline effect observed as MDF were seen in the CS and OS-HA7 fed, saline treated rats. The greatest number of MDF was observed in CS fed, saline treated, antibiotic administered rats (CS-s-Ab). All CS fed rats developed MDF, regardless of their AOM or saline treatment and with or without antibiotic treatment. A significant diet effect was observed in the antibiotic objective as zero MDF were found in SA-HA7 fed rats. No MDF were observed in any mouse. The unusual results from the saline and antibiotic treated rats call into question the reliability of MDF as a biomarker for CRC. Additional research should be conducted to find a more reliable, defined biomarker to better aid in preventative research efforts.

Beta-catenin accumulated crypts (BCAC) is the newest potential biomarker of interest. A dysfunctional or mutated tumor suppressor gene, APC, leads to accumulation of beta catenin, a protein involved in the Wnt pathway. Beta-catenin alters the regulation of the Wnt pathway by allowing uncontrolled cell proliferation which is a key characteristic of carcinogenesis [10, 14]. Our mouse study attempted to analyze and characterize BCAC as a new biomarker by using a novel whole mount immunohistochemistry method instead of a traditionally microsectioned approach. Unfortunately, our efforts were unsuccessful as immunohistochemistry reagents bound

nonspecifically and made analysis unreadable. Further studies should be conducted to investigate BCAC as a new preneoplastic lesion.

In conclusion, our studies suggest that rodents fed starches with moderate resistant contents (OS-HA7 and HA7) showed greatest inhibition of ACF and MDF lesions. Our initial hypothesis stated that animals fed the SA-HA7 diet with the highest resistant content would show the greatest inhibition of preneoplasia when induced with AOM. SA-HA7, however, enhanced ACF and MDF production in both rat and mouse models and therefore our hypothesis was erroneous. The great degree of variation observed between individual animals mandates further animal studies investigating preneoplastic lesions. ACF and MDF are early-stage biomarkers and, as stated before, may not actually progress towards cancer. Future long term studies are necessary to determine relevance of currently known biomarkers. A reliable biomarker for CRC could benefit humans by allowing for earlier detection. This early detection could allow for immediate intervention by change of diet and lifestyle factors, decreasing CRC risk and potentially decreasing the incidence of new cases. In addition to a reliable biomarker, it is also necessary to analyze the long-term effect of dietary resistant starch on colon health and inhibition of colon tumors and cancer.

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APPENDIX A: NOMENCLATURE

ACF	ABERRANT CRYPT FOCI
AOM	AZOXYMETHANE
APC	ADENOMATOUS POLYPOSIS COLI GENE
BCAC	BETA-CATENIN ACCUMULATING CRYPTS
CRC	COLORECTAL CANCER
CS	CONTROL STARCH DIET
DMH	1, 2-DIMETHYLHYDRAZINE
DSS	DEXTRAN SODIUM SULFATE
FAP	FAMILIAL ADENOMATOUS POLYPOSIS
HA7	HIGH-AMYLOSE VII CORNSTARCH DIET
HNPCC	HEREDITARY NONPOLYPOSIS COLORECTAL CANCER
MDF	MUCIN DEPLETED FOCI
MMR	MISMATCH REPAIR
MSI	MICROSATELLITE INSTABILITY
OS-HA7	OCTENYL SUCCINIC-HA7 CHEMICALLY MODIFIED DIET
RS	RESISTANT STARCH
SA-HA7	STEARIC ACID-HA7 COMPLEXED DIET
SCFA	SHORT CHAIN FATTY ACID

APPENDIX B: AUTHORED STANDARD OPERATING PROCEDURES

STANDARD OPERATING PROCEDURE

Title: Safety protocol for AOM handling and disposal and general safety

Lab group: Birt lab

Updated by: Bridget Nelson 4-9-13

Procedure Overview:

A/J mice are injected with AOM once a week for 4 weeks by intraperitoneal injection to induce ACF and BCAC expression. The dietary compound of interest, namely resistant start, is mixed with AIN-93 diet and fed to the mice for 10 weeks starting the third day after the last AOM injection. After the 10 weeks of experimental diet feeding, the whole colon will be collected, cut open, and stained and analyzed for ACF number and multiplicity. Direct Immunohistochemistry will then be performed using a primary antibody for Beta-catenin to analyze and quantify beta catenin expression in colon sample.

Safety Precautions:

Animal handling: Lab coat, gloves, and shoe covers should be worn when working in animal room.

AOM dilutions and mice injections: Lab coat and gloves should be worn when handling AOM. AOM dilution and injections should be conducted in a fume hood. AOM contaminated wastes including syringes, pipets, etc. must be collect in red biohazard

waste bags and put in biohazard–labeled containers and picked up by EH&S. Needles and other sharps should be put in sharps containers and placed into red biohazard bags to be picked up by EH&S.

Special procedures (required when using select carcinogens, reproductive toxins, or substances with a high degree of acute toxicity). Check completed items:

- ☒ Prior approval granted
- ☒ Designated area of use established
- ☒ Containment devices and personal protective equipment available
- ☒ Procedures for the safe removal of contaminated waste established
- ☒ Appropriate decontamination procedures developed
- ☒ Personnel are trained to work with these chemicals

Written by: Bridget Nelson

Date: 04/09/13

Approved by: Diane Birt (Lab Supervisor) Date: 04/10/13

STANDARD OPERATING PROCEDURE

Title: Starch cooking by water boiling method and diet preparation

Lab group: Birt lab

Updated by: Bridget Nelson 5-31-13

The following SOP outlines the methods for cooking starches and diet preparations.

Starch and Ingredient Storage:

Starches are stored in closed tubs and stored at 40°F in the HNSB 2007 walk in cooler.

Diet ingredients are sealed and stored at 40°F in the HNSB LAR diet ingredients coolers.

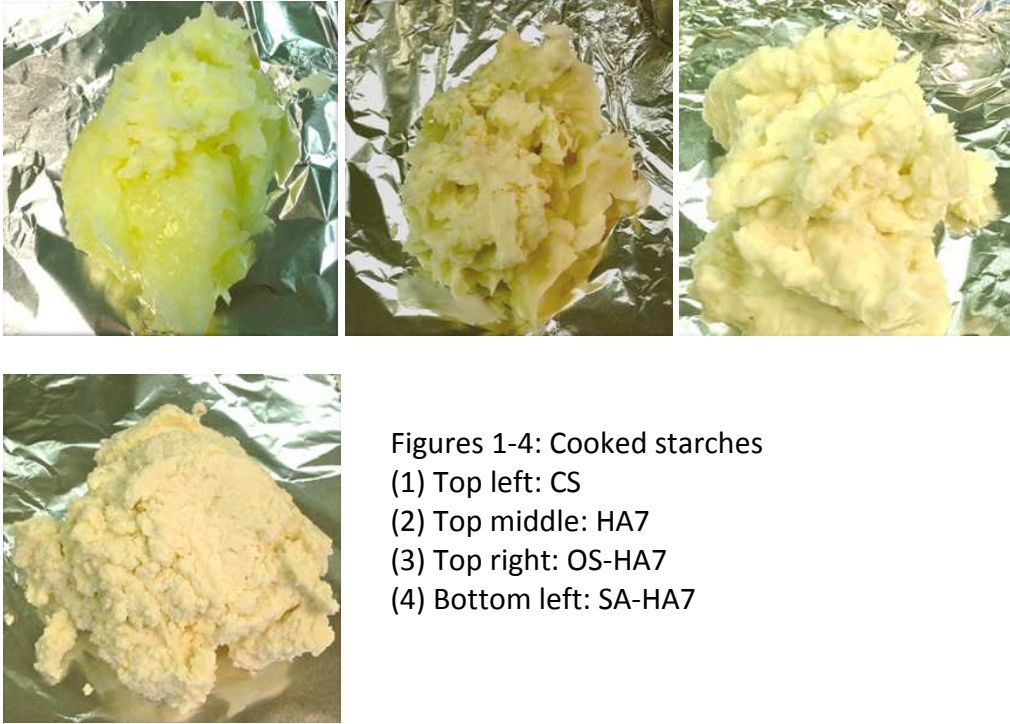
Choline and corn oil are stored at room temperature.

Starch Cooking:

Cooking processes conducted in 2007 HNSB. Starch needed is weighed in a stainless steel pot. Three parts water are added for every part of starch weighed out (1 part starch:3 parts water). The OS-HA7 starch uses 3 parts water minus 100ml. A wooden spoon is used to constantly stir the starch over medium to high heat until starch is cooked to the final endpoints, noted below (Figures 1-4). Note that a plastic spoon will melt. Once the final cooked form is observed, the starch is placed onto labeled pieces of aluminum foil.

Cooking times vary between starch types and depend on the amount of starch being cooked. Generally, CS cooks fastest, followed by OS-HA7, SA-HA7, and then finally HA7. HA7 burns quickly and needs to be cooked at a lower temperature setting (medium low

to medium). The cooked starches are then transported to the HNSB LAR diet preparation room.



Figures 1-4: Cooked starches

(1) Top left: CS

(2) Top middle: HA7

(3) Top right: OS-HA7

(4) Bottom left: SA-HA7

Diet Preparation:

Diet ingredient amounts are calculated based on the AIN-93 diet formulation. If the amount of starch made is less than 500g, mixing by hand is preferred over the standard mixer. All dry ingredients (not starch) are measured and poured into a plastic mixing bowl and mixed. Corn oil is measured last and added last. All ingredients are mixed until homogeneous and the final product is dough-like. Trays are covered with tin foil and labeled with the diet name, lab name, and date prepared for each diet. The diet dough mixture is placed onto the foil covered trays and pressed to various thicknesses: CS-1 inch, HA7-1-1.5", OS-HA7-2-2.5", SA-HA7-1-1.5". The diets are left on metal shelves overnight with a fan on a medium setting directed at the shelves.

STANDARD OPERATING PROCEDURE

Title: Analysis of colonic preneoplastic lesions by general stain and immunohistochemistry methods

Lab group: Birt lab

Updated by: Bridget Nelson 6-3-13

The following SOP outlines the methods for colon staining and analysis.

General staining for ACF and MDF:

1. Remove colon from formalin and remove colon from between microscope slides.
2. Grasp colon gently with forceps and rinse with distilled water.
3. Place colon into 1% alcian blue for 7 minutes.
4. Rinse colon with distilled water.
5. Place colon into the counter stain 1% neutral red for 30 seconds.
6. Rinse colon with distilled water.
7. Lay colon flat on a new microscope slide with the internal slide face up.
8. Place a cover slip over the colon sample and observe under microscope at 4-40x objectives for ACF and MDF (Figures 1 and 2).

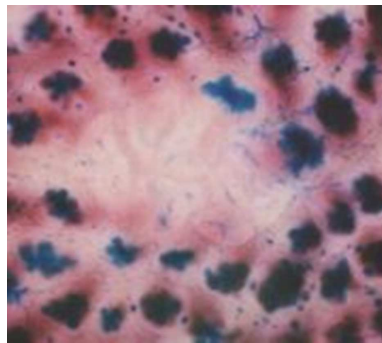
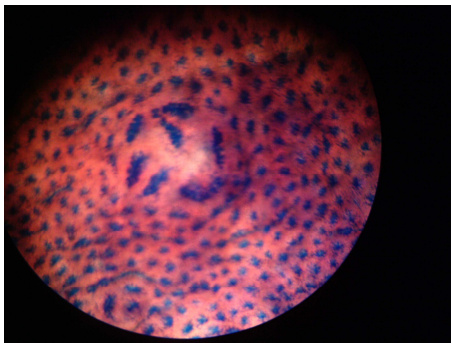


Figure 1 (left): ACF after staining

Figure 2 (right): MDF after staining

Immunohistochemistry for BCAC:

1. After colon section has been analyzed for ACF/MDF, place the colon in a microcentrifuge tube containing a destain solution made of 60% absolute ethanol and 40% absolute acetic acid.
2. Rinse with distilled water and place into 75% ethanol overnight (12-15 hours)
3. Rinse again and place in xylene for 4 hours.
4. Rinse again and place in a peroxidase blocking solution for 5 minutes.
5. Rinse with PBS buffer and place in the antibody solution (diluted to 1:10,000) for 1 hour.
6. Rinse with PBS buffer and place in biotinylated link solution for 30 minutes.
7. Rinse with PBS buffer and place in streptavidin solution for 30 minutes.
8. Rinse with PBS buffer and place in a chromogen solution for 5-20 minutes based on size of colon and wait for colon to change to a darker brown color.
9. Rinse with distilled water and place in counterstain hematoxylin for 1-3 minutes.
10. Rinse with water and dip into ammonia water 5 x and re-rinse with distilled water.
11. Place onto a microscope slide and observe under 4-40x objectives with a microscope (Figure 3).

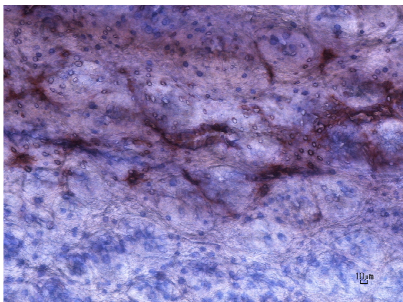


Figure 3: BCAC lesion after IHC staining

STANDARD OPERATING PROCEDURE

Title: Diet feeding for animal studies

Lab group: Birt lab

Written by: Bridget Nelson 6-3-13

The following SOP outlines the methods for feeding animals the resistant starch diets.

1. Weigh and record total weight of each diet before feeding.
2. Based on the total weight, determine the amount that can be fed to each diet group of animals. The average amount of diet per animal per day should be approximately 10-15g, possibly more depending on the batch of mice and individuals within the group.
3. Measure portions of food for each animal based on the amount determined in Step 2 (Figure 1).
4. Weigh and record leftover diet not used to feed animals.
5. Refer to Figures 2-4 for adding diet portions to individual cages.
6. Check back the following day to check the food levels and add more food to animals that are low on food.



Figure 1(left): Measured out food portions for animal feeding.



Figure 2 (right): Diet placed in cages before compression through metal grates.



Figure 3 (left): Compressed diet



Figure 4 (right): Alternate view of compressed diet